

# Patent Application of

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for

## Microbial Production of Nuclease Resistant DNA, RNA and Oligo Mixtures

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention relates to various methods for production of phosphorothioate containing nucleic acids which are generally more nuclease resistant than unsubstituted DNA, RNA, or their oligonucleotides. Micro-organisms or cells are used to catalyze the in vivo synthesis of phosphorothioate nucleic acids by incubation in modified media containing thio-phosphate as a source of phosphorus. The resultant nuclease resistant nucleic acids are useful for the bulk preparation of phosphorothioate double-stranded (ds) DNA and single-stranded (ss) DNA or oligos. Other valuable consequences for the manipulation of cells in vivo include phosphorothioate containing RNAs that are resistant to RNA turnover. These effects are not limited to prokaryotes but occur in eukaryotes as well.

Bacteria infected with recombinant phage DNA and grown in thio-phosphate containing media produce single-stranded phage DNA with phosphorothioate linkages. Recombinant phosphorothioate phage DNA can be further processed in vitro to generate oligo mixtures spanning the entire cloned recombinant DNA insert. Such oligo mixtures are not only nuclease resistant but also have enhanced antisense activity by virtue of multiple target sites embodied by an entire recombinant cDNA or exon phage DNA insert.

Cells incubated in thio-phosphate containing media also incorporate the modified phosphate into the backbone of RNA resulting in an increased resistance of the modified RNA to RNases. Cells grown in 100% thio-phosphate substituted media generate higher yields of mRNA per cell. At lower ratios of thio-phosphate to phosphate many cellular mRNAs are preferentially stabilized over more abundant species resulting in a concomitant increase in the protein synthesis of such mRNAs. The novel mRNA stabilizing media can be used to increase the protein levels of native and/or recombinant proteins in prokaryotes as well as eukaryotes.

100% thio-phosphate substituted media inhibits DNA repair mechanisms and can be used to increase the natural mutation rate, particularly in bacteria. The media can be used to generate new variant strains or plasmids harbored therein with high efficiency by several cycles of growth amplification in modified media.

#### 2. Description of Related Disclosures

Phosphorothioate containing analogues of oligonucleotides are widely used for gene ablation, otherwise known as antisense technology, to diminish gene expression in tissue culture cells or in the treatment of entire organ systems as novel pharmaceutical reagents (J. Murray Ed. (1992) "Antisense RNA & DNA," Wiley-Liss, New York; van der Krol, et al (1988) *BioTechniques* 6:958-975; Clercq, et al (1970) *Virology* 42:421-428). DNA and RNA oligonucleotides can be chemically synthesized in several hundred milligram to gram quantities (Froehler et al (1986) *Nucleic Acids Res.* 14:5399-5407; Sinha et al (1983) *Tetrahedron Lett.* 24:5843-5846; Letsinger et al (1965) *J. American Chem. Soc.* 87: 3526; Sinha and Fry (1984) In Sanghui and Cook (Eds) "Carbohydrate Modifications in Antisense Research," American Chemical Society, Washington, DC), although they are often used on a smaller scale for research purposes. Phosphorothioate oligos are typically prepared via solid phase synthesis and oxidative sulfurization with the Beaucage or Zon reagent. More recent advances include the preparation of

chirally enriched phosphorothioate oligos (Just et al (2000) US Patent 6,031,092; Stec et al (1999) US Patent 5,883,237) as well as large scale synthesis procedures involving solution phase techniques (Yau (1997) US Patent 5,644,048; Ravikumar et al (1999) US Patent 6,001,982).

Long phosphorothioate polymers (>100 bases) can be generated in vitro using RNA or DNA polymerases and the appropriate phosphorothioate analogues of the nucleoside triphosphates as substrates (Griffiths and Eperon (1987) Nucleic Acids Res. 15:4145-4162; Suh and Eperon (1992) Nucleic Acids Res. 20:6303-6309; Eckstein (1985) Annu. Rev. Biochem. 54:367-402). Enzymatic synthesis being more costly, is typically used when smaller quantities suffice or longer polymers are required. Enzymatic synthesis results in the stereospecific incorporation of Sp nucleotides with inversion of configuration to the Rp form in the internucleotidic linkage. In this regard, oligodeoxynucleotides of the Rp configuration form a more stable complex with mRNA and hence may be a better ablator of gene expression (Koziolkiewicz et al (1995) Nucleic Acids Res. 23: 5000- 5005). Several novel in vitro approaches have been devised for the enzymatic preparation of oligonucleotides involving the formation of concatamers and various means of separating these (Lackey et al (1998) US Patent 5,739,311; Kacian et al (1999) US Patent 5,916,777; Dattagupta et al (1999) 5,932,450; Kool et al (2000) 6,096,880).

The utility of antisense oligos for the ablation of gene expression has gained considerable experimental evidence. Typically an antisense oligo hybridizes with its target RNA in a cell and thereby leads to the inactivation of the target transcript. DNA and RNA oligos taken up by cells in culture are rapidly degraded unless they are chemically modified. Phosphorothioate substituted DNA oligos are resistant to nucleases and upon uptake into the cell inhibit gene expression by stimulating an RNase H activity which degrades the RNA component of the mRNA/DNA hybrid formed.

Oligo mixtures have been shown to be more effective in antisense studies than individual oligos (Nieto et al (1994) Science 264:835-839; Morgan et al (1993) Nucleic Acids Res. 21:4615-4620; Dattagupta et al (1998) US Patent 5,739,309) but are not widely used as they are not as readily synthesized. Mixtures of oligos are effective at much lower concentrations and with reduced toxicity. These observations suggest that the rate limiting step for antisense inhibition is the interaction of the oligo with its target mRNA. Mixtures of antisense oligos by sensing a larger mRNA target may be more efficient and thus require lower inhibitory concentrations. An additional advantage of oligo mixtures is reduced toxicity through reduced concentrations and increased specificity. It has been suggested that non-specific effects may result from short stretches of homology between an oligo and another non-target mRNA molecule that may hybridize long enough to be destroyed by RNase H (Wolf (1992) PNAS 89:7305-7309). The use of mixtures of oligos corresponding to different parts of the mRNA to be inactivated should reduce these effects since no one oligo would accumulate to a significant extent leaving the target mRNA as the site of preferred hybridization.

The present invention provides a means for the facile production of phosphorothioate containing oligo mixtures, and DNA or RNA polymers. The method involves the use of bacteria as an economical means to generate milligram quantities of DNase resistant oligo mixtures. Large fermentors could presumably be used to synthesize gram quantities of dsDNA, ssDNA, or oligo mixtures. The nucleic acids generated are stereospecific, corresponding to the preferred more stable Rp configuration for antisense work. To produce antisense oligo mixtures no DNA sequence information is required, simply the orientation of the cloned insert with respect to transcription. For example, cDNA clones that have been inserted unidirectionally into a recombinant vector (Alting-Meese and Short (1989) Nucleic Acids Res. 17:9494-9501) can be manipulated to generate antisense oligos.

Phosphorothioate linkages in RNA are also known to protect such molecules from degradation by RNases found in serum or inside cells (Matzura and Eckstein (1968) European J. Biochem. 3:448-452). The in vivo incorporation of thio-phosphate into the mRNA backbone provides a ready means to induce the stabilization of cellular mRNA. Bacterial mRNAs are very unstable with half-lives on the order of ~3 minutes. Eukaryotic mRNAs are considerably more stable with half-lives that range from 30 minutes to 10 hours or more. To enhance protein synthesis a particular ratio of modified phosphate to phosphate is optimal for a given organism. Following the uptake of thio-phosphate into cells less stable mRNAs are enriched and compete more effectively with bulk stable mRNAs, such as those coding for hydrolytic

enzymes, for protein synthesis. Thus the present invention can be used to increase or induce an increased amount of a desired protein in cells.

In vitro studies have shown that phosphorothioate linkages in DNA molecules prevent DNA editing and repair functions by inhibiting the 5'-3' exonuclease activity associated with DNA polymerase (Burgers and Eckstein (1979) J. Biol. Chem. 254:6889-6893). This leads to a significant loss in fidelity of the enzyme depending on the polymerase used. The present invention provides a means for the in vivo incorporation of thio-phosphate into DNA resulting in an increase in the natural mutation rate, particularly in bacteria. For E. coli the increase in mutation rate is greater than expected from previous in vitro studies indicating that multiple repair mechanisms are blocked. The non-toxic media is safe and easy to use with repetitive cycles of amplification and growth resulting in an increase in desired mutations. The media can be used to generate new strains or to introduce mutations into recombinant DNA molecules with a high frequency after several cycles of amplification.

### SUMMARY OF THE INVENTION

The present invention relies on the in vivo incorporation of thio-phosphate into nucleic acids, introducing nuclease resistant phosphorothioate linkages into both DNA and RNA molecules. Mixtures of oligos spanning a cloned DNA fragment of interest, can be generated by the processing of single-stranded recombinant M13 phage DNAs grown in thio-phosphate containing media. To generate antisense oligo mixtures, the cloned fragment is inserted in the orientation that opposes transcription. M13 recombinant phage substituted with phosphorothioate are purified by standard PEG (polyethyleneglycol) precipitation. The insert is then processed to generate oligonucleotides by enzymatic or physical means.

RNA molecules with thio-phosphate linkages are more resistant to RNases. The in vivo incorporation of thio-phosphate into RNA can be used to manipulate the mRNA stability of genes and thereby enhance the protein synthesis of specific populations. Maximal protein synthesis is achieved by partial substitution of the RNA backbone. Generally less stable and more typical mRNAs are enriched relative to very stable mRNAs resulting in their preferential accumulation and translation. Growth in thio-phosphate containing medias allows for the selective enhancement of protein synthesis in both prokaryotes and eukaryotes.

The presence of phosphorothioate linkages in bacterial DNA inhibits normal cellular DNA repair mechanisms resulting in a greatly enhanced mutation rate (Goodman et al (1993) Crit. Rev. Biochem. Mol. Biol. 28:83-126). Recombinant M13 phage DNA containing the beta-galactosidase gene exhibit a mutation rate for beta-galactosidase of one in every four hundred plaques after one cycle of infection and amplification in thio-phosphate media. After two cycles of phage infection and amplification the mutation frequency for the beta-galactosidase enzyme increases to one in six plaques. The media can be used to generate mutations, particularly, in prokaryotes in a relatively safe manner compared with other known mutagens. Eukaryotes are not as readily affected owing to more efficient repair mechanisms.

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 demonstrates the resistance of M13 phage DNA grown in thio-phosphate media to DNase I digestion. Lanes C and P are control lanes without DNase for normal and thio-phosphate substituted DNA. The two forms of phage DNA indicated by arrows: closed circular (I) and linear (II). Conversion of form I to II during DNase digestion was assayed at 1, 2, and 3 hrs. (lanes 1-3 and 4-5, respectively). By two hrs. (lane 2) form I is completely digested in normal phage DNA samples. In contrast, using DNA from cells grown in thio-phosphate media, no conversion is detected for up to 3 hrs. of incubation (lanes 4-6).

FIG. 2 demonstrates the resistance of *E. coli* RNA from cells grown in thio-phosphate media to RNase digestion. Total RNA was prepared using Quigley and Holmes rapid boiling procedure (see text) to lyse cells and which typically leaves low molecular weight RNA species (lane 1 arrow). The RNA was separated on a non-denaturing agarose gel along with a ds DNA molecular weight marker (50 bp-2 Kb, Bio-Rad). The addition of RNase (1/5, 1/10, 1/20 dilutions of 1 mg/ml; lanes 2-4 respectively) resulted in the disappearance of normal RNA. Lane 5 (arrowhead) shows high molecular weight RNA detected only from cells grown in thio-phosphate media. The RNA is also more resistant to RNase as shown in lanes 6 and 7 using no dilution or a 1/5 dilution of RNase (1 mg/ml).

FIG. 3 demonstrates the stabilization and normal RNA processing of yeast actin mRNA from cells grown in thio-phosphate containing medias. Total RNA was isolated from cells grown in medias with various ratios of thio-phosphate to normal phosphate and used for Northern analysis. Marker corresponds to biotinylated QX174/Hinf digest (151-726 bp). Lanes 1-4 correspond to RNA from cells grown at ratios of 100%, 50%, 20%, and 0% thio-phosphate to phosphate. Arrow indicates band of the expected MW for mature mRNA with intron removed visible in lane 1 using ~1 ug of total RNA from 100% substituted media. Trace amounts of a larger processing intermediate can also be discerned (lane 1).

FIG. 4 depicts a table summarizing the results of varying ratios of thio-phosphate to normal phosphate on gene specific expression in cultured organisms. A 100% ratio represents maximal substitution of the media with thio-phosphate. Arrows pointing upward represent enhanced expression relative to normal media whereas those pointing downward represent diminished expression. Thicker arrows correspond to a greater degree of change than thinner arrows. The approximate fold enhancement is indicated next to the arrow at peak expression.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

Organisms require a metabolically useful source of phosphorus for DNA replication and continued growth. In many instances inorganic phosphate is used by cells as a source of phosphate in the synthesis of nucleic acids and/or the modification of cellular proteins, etc. The sulfated version of inorganic phosphate or thio-phosphate is also absorbed and utilized by cells in the synthesis of nucleic acids. The modified phosphate is presumably incorporated into dNTP and NTP precursor pools. Generally to achieve high levels of thio-phosphate incorporation it is necessary to remove all sources of inorganic phosphate from the culture media. For bacterial cells and yeast, minimal medias or chemically defined medias are easily prepared using thio-phosphate instead of phosphate. In other instances it may be useful to deplete nutrient broths of phosphate prior to use. This can be done by magnesium sulfate precipitation in the presence of ammonium hydroxide (Rubin (1973) *J. Biol. Chem.* 248:3860). For invertebrate and vertebrate cells the growing use of serum free medias makes it readily possible to substitute thio-phosphate as a source of phosphorus for these cells as well (Jakoby and Pastan, Eds. (1979) *In Methods in Enzymology "Cell Culture,"* Vol. 58; Cell Systems, Inc.).

Bacterial cells respond well to high levels of thio-phosphate producing high yields of recombinant DNA molecules when grown in such medias. The modified phosphate appears to be taken up by cells as readily as inorganic phosphate. The use of a mixture of thio-phosphate and inorganic phosphate results in intermediate levels of thio-phosphate incorporation. It is also possible to first deplete bacterial cells of internal inorganic phosphate by growing the cells in minimal media using glycerol-phosphate as a source of phosphorus. In the absence of organic phosphate a phosphatase is induced which hydrolyzes phosphate esters, such as glycerol phosphate without leading to significant phosphate accumulation.

The preparation of large quantities of thio-phosphate DNA can be readily achieved using bacterial cells and recombinant vectors such as M13. Propagation of M13 bacteriophage in thio-

phosphate media results in ssDNA yields similar to phage grown in normal media (5-10 ug/ml of culture). Inserts up to 1 Kb are typically cloned into M13 (Messing (1981) *Nucleic Acids Res.* 9:309-317). When inserted in the antisense orientation, antisense DNA oligo mixtures can be obtained. It may be of interest to purify recombinant cDNA sequences from vector sequences. There are several ways in which this may be achieved. The most precise method involves the use of restriction enzymes to cleave the recombinant DNA insert. However, the ability of a particular restriction enzyme to cleave thio-phosphate substituted DNA must be considered. Many enzymes are retarded or blocked by the presence of a phosphorothioate linkage (Taylor et al (1985) *Nucleic Acids Res.* 14:8749-8764). As a rule four base pair recognition sequence enzymes appear to be less sensitive to the presence of thio-phosphate than six base pair recognition enzymes. Several of the six base pair cutters that are more useful include: Pvu II, Sac I, Sal I, Sma I, Mst II, Hgi AI. Other restriction enzymes that can be used with excess enzyme and prolonged incubation include: Bam HI, Eco RI, Bgl I, and Hind III. Partial cleavage of single-stranded phosphorothioate DNA has also been observed with other enzymes (Lackey et al (1998) US Patent 5,739,311). An alternative approach would be to sonicate or degrade the phage DNA and selectively extract the insert sequences by hybridization with excess minus strand M13 phage DNA attached to magnetic beads or solid phase supports.

After the cDNA insert is purified as required it is necessary to degrade the single-stranded DNA to suitably sized oligos. Many physical methods have been employed to fragment DNA, these include sonication, french press, wharing blender, or hydroshear (Oefner et al (1996) *Nucleic Acids Res.* 24:3879-3886) (Davis et al (1973) *Methods in Enzymology* Vol. 21:378-381). It is also possible to degrade thio-phosphate single-stranded DNA with S1 nuclease or DNase I. Normally DNase cleaves double-stranded DNA more readily than single-stranded DNA when magnesium is used in the buffer. However, if the magnesium is replaced with manganese then the efficiency increases (Melgar and Goldthwaite (1968) *J. Biol. Chem.* 243:4409-4417). The rate of digestion of single-stranded phosphorothioate DNA is still, however, much slower than unsubstituted DNA. DNase I digestion, however, is easy to control by varying the amount of enzyme. With prolonged incubation DNase I will degrade phosphorothioate single-stranded M13 phage DNA into oligos less than 50 bp in length. Previous assays used to measure DNase digestion of phosphorothioate nucleotides were not sensitive enough to detect digestion (Spitzer and Eckstein (1988) *Nucleic Acids Res.* 16:11691-1170). Furthermore, studies indicating stereoselectivity of S1 for short phosphorothioate nucleotides do not hold for much longer phosphorothioate polymers (Eckstein (1985) *Annu. Rev. Biochem.* 54:367-402).

Oligo mixtures have been used in antisense studies where the oligos were micro-injected into frog oocytes (Morgan et al (1993) *Nucleic Acids Res.* 21:4615-4620). Such mixtures were much more effective than any one oligo alone. This can be explained by assuming that the rate limiting step is the initial interaction between the oligo and target mRNA. Presently, oligos are used at concentrations that are in far excess of a one to one ratio of oligo to target even after accounting for the poor uptake of oligos by cells (1-10%). By increasing the mRNA target sites one might expect according to the poisson distribution an exponential increase in effective interactions resulting from the increase in density of target sites. If one assumes a typical mRNA size such as a 1.2 Kb mRNA and an average oligonucleotide size of 30 bp then the number of targets equals  $N-n+1$  where N is 1200 and n is 30. Therefore, a potential decrease in concentration required of at least several orders of magnitude can be expected. Even mixtures of two oligos have been shown to be more effective together at ten fold lower concentrations than either one alone (Nieto et al (1994) *Science* 264:835-839). However, in general and particularly for cell culture or in vivo studies DNase resistant oligos are required. It is not practical to synthesize a mixture of 1000 oligos. Therefore, the present method provides a ready means to generate oligo mixtures that are DNase resistant.

Many applications can be envisioned for the use of large gene encoding DNase resistant molecules. Both double-stranded and single-stranded molecules can be produced with thio-phosphate linkages. Such nuclease resistant linkages should help to stabilize DNA molecules used in cells or in vivo such as recombinant DNA vaccines (Wolff et al (1990) *Science* 247:1465-1468) or expression constructs. Many bioprocessing and diagnostic procedures involve the contact of cell extracts with purified DNAs.

DNase resistant molecules should exhibit greater stability than unmodified molecules with similar selectivity. More recently it has been shown that certain CG sequences found in bacteria illicit a strong immune response which is potentiated by phosphorothioate linkages (Weiner et al (1997) PNAS 94:10833-10837). Thus bacterial DNAs modified with phosphorothioate backbones have the potential to be strong adjuvants.

Medias substituted with 100% thio-phosphate can be used to increase the mRNA content per cell and thereby facilitate northern, RT-PCR, or other subsequent analyses. While full-strength medias provide the maximum stabilization of mRNA it must be kept in mind that at such levels RNA processing or regulatory mechanisms may be impaired (Suh et al (1992) Nucleic Acids Research 20:6303-6309). In this regard, since both bacteria and yeast grow readily in 100% thio-phosphate media such processing if impaired must not be necessary for viability. Furthermore, yeast cells grown in 100% thio-phosphate media and are able to splice introns for such genes as actin (Fig. 3).

It is possible to increase the protein synthesis levels of many mRNAs through selective stabilization at optimal thio-phosphate to phosphate ratios. At very high ratios of thio-phosphate to phosphate total protein synthesis declines. At lower ratios where total protein synthesis is not affected, the levels of many typical and less abundant species is increased presumably at the expense of the most abundant species. The process of enhancement results from the preferential accumulation of proteins encoded by less stable mRNAs. The ratio of thio-phosphate to phosphate rather than the total phosphate level confers the degree of stabilization. In yeast it is known that many mRNAs for extracellular hydrolytic enzymes such as amylase are atypically stable with half-lives more than ten hrs. In contrast, most yeast mRNAs have half-lives on the order of 20 mins. Very low ratios of thio-phosphate to phosphate (10%) in yeast enhance the accumulation of the more typical mRNAs relative to the abundant and stable hydrolytic mRNA population. In bacteria, similar to yeast, beta-galactosidase is at least one abundant hydrolytic species that is reduced under conditions leading to an increase of other gene products. The degree of protein enhancement depends somewhat on the relative stability of corresponding mRNAs but can be as much as ten fold for less stable or heterologous gene products in bacteria and/or yeast.

Fermentation procedures include methods to generate proteins and enzymes or bio-catalytic processes in which microorganisms are used or manipulated to catalyze reactions in the generation of chemicals such as organic acids, antibiotics, vitamins, amino acids, etc. The ability to modulate mRNA levels may greatly enhance the production of desired products. The degree of enhancement can be controlled by the ratio of thio-phosphate to phosphate used as well as through the addition of the modified phosphate at various time points during the fermentation process. In this regard, individual organisms vary in their requirement for phosphate. The optimal ratio of thio-phosphate to normal phosphate that enhances protein synthesis of a given gene product must be determined for a given organism. The optimal ratio may also vary under different cellular physiological states such as that imparted by the vector system employed (i.e. plasmid or phage) or growth phase (trophophase versus idiophase). While a generally optimal ratio for most genes may emerge for a given organism it is still possible that an unusually unstable mRNA may be preferentially induced at lower ratios of thio-phosphate to phosphate levels. If the organism can tolerate an increase in total cell protein then even stable mRNAs may be enhanced over time.

For bio-catalytic reactions there are several ways in which increased mRNA stability could be used to increase production. (It should be remembered that while increased mRNA levels generally result in increased protein levels of a given gene, this is not always the case.) For a particular process enhancement may arise through overcoming the rate limiting step(s) in the bio-reaction sequence. If the product is synthesized during the growth or the trophic phase then there are a number of possible points which may be rate limiting such as glucose uptake, glycolysis (often very fast), Krebs' cycle, enzymatic catalysis or product feedback inhibition. Thus the rate of fermentation, duration, and/or yield of product may be increased depending on which step is rate limiting. If the product is produced during the idiophase as a secondary metabolite then mRNA stabilization of catalytic enzymes may also enhance yields during this later stage of fermentation. During the idiophase the modified phosphate can be simply added to the

media to enhance mRNA stabilization. It is important to keep in mind that the synthesis of many secondary metabolites is regulated by phosphate. This does not pose a problem since it is the ratio and not the level of phosphate that is important for stabilization. However, if thio-phosphate is added at late stages to the medium the maximum tolerated amount should not be exceeded. Alternatively, phosphate deregulated mutants may be employed (Martin (1977) Adv. Biochem. Eng. 6:105-127).

Use of thio-phosphate as described here is not limited to traditional culture systems. Many products are obtained from multi-cellular organisms and plants as well as transgenic versions of these. Modified feed, injection, or submersion of aquatic species in conditioned media can be envisioned as meets all environmental, health, and humane treatment regulations.

The mutagenesis of cells or specific gene segments is an important tool in the field of biotechnology. Expanding the power of mutagenesis is the technique of PCR shuffling that is used to generate new combinations of mutant alleles for specific genes (Stemmer (1994) PNAS 91:10747-10751). The method requires the introduction of new mutations which serve as a source of genetic diversity for subsequent recombination events. The present system of growing cells harboring recombinant plasmids or phage of interest in modified media to increase the natural mutation rate in bacteria can also be used to generate genetic diversity for PCR shuffling and other applications. The method is relatively inexpensive and is not limited by the size of the gene as compared with in vitro methods. The approach is also much simpler to employ than mutator strains (Greener et al (1996) Methods Mol. Biol. 57: 375-385). The new approach to mutagenesis has the additional advantage that cells or cells harboring recombinant plasmids can be grown under selective conditions.

## EXPERIMENTAL

### Example I

#### Method to generate antisense oligo mixtures.

The propagation of M13 phage in thio-phosphate containing media first requires the cultivation of the appropriate host strain such as JM109 which requires minimal media to select for the F' pillus. Minimal plates are prepared as follows: bactoagar, 10.5 g/L;  $K_2HPO_4 \cdot 3H_2O$ , 4.5 g/L;  $KH_2PO_4$ , 4.5 g/L;  $(NH_4)_2SO_4$ , 1 g/L; sodium citrate  $2H_2O$ , 0.5 g/L; Adjust pH to 7.4 and autoclave. Then add the following:  $MgSO_4 \cdot 7H_2O$ , 0.2 g/L (sterilized separately as a conc. solution); (thiamine HCL, 5ug/L; glucose, 4 g/L sterilized separately by filtration). Glucose can also be sterilized by autoclaving separately.  $FeCl_2$  (500 ug/L) can also be added as needed.

Thio-phosphate containing media is prepared similarly as minimal media except that the inorganic phosphates are replaced with thio-phosphate ( $Na_3SPO_3 \cdot XH_2O$ ) 10- 15 g/L and KCL (1.5 g/L). Thio-phosphate contains variable amounts of water (10-15 per molecule) not included in molecular weight calculations. It is almost 50% water by weight. Note pH control is important in maximizing thio-phosphate stability. To ensure adequate growth, use a high density innoculum. The preferred pH is neutral or slightly basic. For media to deplete cellular phosphate pools, beta-glycerolphosphate is substituted for inorganic phosphates at a concentration of ~25 g/L.

Several media are required for the production of infectious phage particles: LBM medium (bacto tryptone, 10 g/L; bacto yeast extract, 5 g/L; NaCl, 5 g/L;  $MgCl_2 \cdot H_2O$ ; 10 mM Tris/HCL pH 7.5. LBM agar plates (add 15 gm Bacto-agar to 1 liter of LBM medium and autoclave); soft agar (add 7 gm of bacto-agar to 1 liter of LBM medium. Store at 4°C and heat to 45°C before use.).

Phage are generated by transforming JM109 cells with the replicative form of M13 DNA or double-stranded DNA using the calcium chloride (Dagert and Ehrlich (1979) Gene 6:23) or DMSO/PEG (Chung et al (1989) PNAS 86:2172-2176). The transformed cells produce infectious particles when grown in nutrient broth. To .3 ml of competent cells add 5 ng of DNA and let the mixture sit on ice for 40 min. Then heat shock the cells at 42°C for 2 min. and add the following: .2 ml of fresh JM109 cells and 3



ml of top agar at 45°C. Mix and plate directly onto LBM plates. Let the plates solidify and then incubate at 37°C until plaques are seen (overnight). The plaques appear as turbid clearings on the bacterial lawn. A plaque can then be picked with a sterile toothpick and used to inoculate 2 ml of LBM broth and grown with shaking overnight. The cells are spun out and the supernatant is saved as phage stock at 4°C. The supernatant (20 ul) can be run directly on a gel to test for the presence of DNA. The titer of the stock should be checked to ensure high yields. The titer should be at least  $1 \times 10^{10}$ /ml.

To prepare phosphorothioate phage substituted DNA, JM109 cells are incubated overnight in LB broth. The starter culture can be used directly or spun out and washed with thio-phosphate media to remove phosphate present in the yeast extract of LBM broth. Generally 500 ml of thio-phosphate containing media are inoculated with 10 – 25 ml of overnight culture (high density) and grown for ~1.5 hrs to an OD 600 = .3 (early log phase). At this point infect cells with phage stock at a moi of 1 pfu per 10 bacterial cells. This corresponds to approximately 500 ul of phage stock. The cells are then incubated at 37°C with shaking for 3 hr. and not more. For intermediate levels of thio-phosphate, cells are incubated in media containing an equal mixture of thio-phosphate and inorganic phosphate.

M13 phage DNA can be purified by standard PEG precipitation (ppt). Cells are pelleted in a centrifuge at 12,000 X g and the supernatant is saved. 125 ml of 2.5 M NaCl, 20% PEG 6000 is added to each 500 ml of phage supernatant. Let sit at room temperature or overnight in the refrigerator. Spin sample for 20 min. at ~12,000 X g. Suck off all the supernatant, respin briefly to get drops off the wall of the tube and remove all liquid. Dissolve in TE (10 mM Tris-HCL, pH 8.0, 1 mM EDTA) buffer. Add 2% SDS and heat at 65°C for 10 min. To remove PEG perform a phenol/chloroform extraction and several chloroform extractions as necessary, until the interface is clear. Ethanol ppt the aqueous layer, pellet and resuspend in sterile water. Traces of genomic DNA and RNA can be removed with DNase (standard magnesium buffer: 50 mM Tris pH 7.5, 1mM MgCl<sub>2</sub>, 100 units/ml) and RNase (10 ug/ml) respectively at 24°C for ~10 min. Chloroform extract and then ethanol ppt. sample.

To process the cloned insert restriction enzymes are used that flank the insert EcoR I and Sal I can be used with mp18 in a suitable buffer (6 mM Tris pH 7.9, 6 mM MgCl<sub>2</sub>, 150 mM NaCl, 1 mM dithiothreitol). The sample is then run in a non-denaturing agarose gel and the insert excised from the gel. The insert can then be purified by electro-elution.

To fragment the insert DNA the sample is treated with DNase in the presence of manganese (5 mM manganese, 50 mM Tris-HCL, pH 7.5) (Melgar and Goldthwaite (1968) J. Biol. Chem. 243:4409-4417). Use 200-500 units of enzyme per ml and digest overnight at 37°C. The DNase can then be removed by chloroform extraction.

To assay for thio-phosphate incorporation resistance to DNase digestion can be monitored using manganese containing buffer. When the phage DNA sample is run on a high resolving agarose gel the closed circular form is separated from the nicked linear form. The disappearance of the closed circular form can be monitored by incubating an aliquot with 2 units of enzyme in a ten ul reaction for 1-3 hrs. By two hrs. the closed circular form disappears in normal DNA preparations. Modified DNAs are more resistant depending on the ratio of thio-phosphate to phosphate used in the growth medium (Figure 1). The closed circular form of DNA disappears by 3 hrs. when a 50% ratio of thio-phosphate to phosphate is used compared to the persistence of the circular form when the growth medium contains 100% thio-phosphate. Other traditional methods to examine phosphorothioate nucleotides include <sup>31</sup>P NMR (Eckstein and Jovin (1983) 22:4546-4550).

## Example II

### Method of stabilizing RNA or DNA in vivo and in vitro.

Growth of cells in thio-phosphate containing media results in the incorporation of thio-phosphate nucleotides into RNA as well as DNA. The resultant RNAs accumulate and are protected from degradation both in vivo and in vitro during subsequent isolation of total RNA. Both E. coli and yeast are



easily grown in minimal medias with thio-phosphate as the only source of phosphorus. *S. cerevisiae* (Baker's yeast, ATTC #7754) grow in essential minimal media with no phosphates (EMM [Contents/L: 3 g phthalic Acid, K<sup>+</sup>, 5 g NH<sub>4</sub>Cl, 20 g dextrose, 1.05 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 14.7 mg CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 g KCl, 0.04 g Na<sub>2</sub>SO<sub>4</sub>, 1 mg panthothenic acid, 10 mg nicotinic acid, 10 mg myo-inositol, 1 mg biotin, 0.5 mg boric acid, 0.4 mg MnSO<sub>4</sub>, 0.4 mg ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.2 mg FeCl<sub>2</sub>·6H<sub>2</sub>O, 40 ug molybdc, 0.1 mg KI, 40 ug CuSO<sub>4</sub>·5H<sub>2</sub>O, 1 mg citric acid], BIO101) and supplemented with thio-phosphate (1-10 g/L) as well as each of the following at 50 mg/L adenine, histidine, leucine, lysine, and uracil (Sp, BIO101). For bacteria see above minimal media preparation.

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Yeast RNA is more susceptible to degradation and care must be taken to remove all RNases. Yeast RNA can be isolated by methods involving the lysis of cells in the presence of guanidinium salts (TRIzol, MRC; Kadmar and Evans (1992) BioTechniques 12:632-637). Briefly cells are pelleted and an equal volume of acid washed glass beads (Sigma Chemical) added. To this sample a small amount of TRIzol is added to initiate lysis. The sample is pipetted up and down while on ice. The sample is then vortexed for 40 sec and placed on ice before vortexing once again. The volume of TRIzol is then increased such that for each ~100 mg of cells, 1 ml of TRIzol is added. Then 0.2 ml of chloroform is added to separate the aqueous and organic phases. After centrifugation the aqueous layer is ppt with isopropanol.

Northern analysis of actin mRNA indicates that significant stabilization of mRNA occurs in 100% thio-phosphate media. The mRNA is in the size range expected for a mature mRNA in which the intron sequences have been removed (Figure 3). A larger less prominant molecular weight species is also detected suggesting the accumulation of processing intermediates. The actin probe is prepared by amplification of yeast genomic DNA (act1, YFL039C, Saccharomyces Genome Database <http://genome-www2.stanford.edu>) with the following PCR primers:

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Thio-phosphate can also be utilized by whole animals and/or complex tissues. *Carassius auratus* incorporates thio-phosphate into the DNA of intestinal cells; these cells are known to turnover in adults. Fish can be maintained in distilled water with NovAqua and AmQuel conditioners (US Patent 4,666,610). Goldfish flakes are also added for food. Normal phosphate levels in the aquarium are less than 1 mg/L (Hanna Phosphate Test Kit; Hanna Instruments). Fish are transferred to 100 ml of

ml of top agar at 45°C. Mix and plate directly onto LBM plates. Let the plates solidify and then incubate at 37°C until plaques are seen (overnight). The plaques appear as turbid clearings on the bacterial lawn. A plaque can then be picked with a sterile toothpick and used to inoculate 2 ml of LBM broth and grown with shaking overnight. The cells are spun out and the supernatant is saved as phage stock at 4°C. The supernatant (20 ul) can be run directly on a gel to test for the presence of DNA. The titer of the stock should be checked to ensure high yields. The titer should be at least  $1 \times 10^{10}/\text{ml}$ .

To prepare phosphorothioate phage substituted DNA, JM109 cells are incubated overnight in LB broth. The starter culture can be used directly or spun out and washed with thio-phosphate media to remove phosphate present in the yeast extract of LBM broth. Generally 500 ml of thio-phosphate containing media are inoculated with 10 – 25 ml of overnight culture (high density) and grown for ~1.5 hrs to an OD 600 = .3 (early log phase). At this point infect cells with phage stock at a moi of 1 pfu per 10 bacterial cells. This corresponds to approximately 500 ul of phage stock. The cells are then incubated at 37°C with shaking for 3 hr. and not more. For intermediate levels of thio-phosphate, cells are incubated in media containing an equal mixture of thio-phosphate and inorganic phosphate.

M13 phage DNA can be purified by standard PEG precipitation (ppt). Cells are pelleted in a centrifuge at 12,000 X g and the supernatant is saved. 125 ml of 2.5 M NaCl, 20% PEG 6000 is added to each 500 ml of phage supernatant. Let sit at room temperature or overnight in the refrigerator. Spin sample for 20 min. at ~12,000 X g. Suck off all the supernatant, respin briefly to get drops off the wall of the tube and remove all liquid. Dissolve in TE (10 mM Tris-HCL, pH 8.0, 1 mM EDTA) buffer. Add 2% SDS and heat at 65°C for 10 min. To remove PEG perform a phenol/chloroform extraction and several chloroform extractions as necessary, until the interface is clear. Ethanol ppt the aqueous layer, pellet and resuspend in sterile water. Traces of genomic DNA and RNA can be removed with DNase (standard magnesium buffer: 50 mM Tris pH 7.5, 1mM MgCl<sub>2</sub>, 100 units/ml) and RNase (10 ug/ml) respectively at 24°C for ~10 min. Chloroform extract and then ethanol ppt. sample.

To process the cloned insert restriction enzymes are used that flank the insert EcoR I and Sal I can be used with mp18 in a suitable buffer (6 mM Tris pH 7.9, 6 mM MgCl<sub>2</sub>, 150 mM NaCl, 1 mM dithiothreitol). The sample is then run in a non-denaturing agarose gel and the insert excised from the gel. The insert can then be purified by electro-elution.

To fragment the insert DNA the sample is treated with DNase in the presence of manganese (5 mM manganese, 50 mM Tris-HCL, pH 7.5) (Melgar and Goldthwaite (1968) J. Biol. Chem. 243:4409-4417). Use 200-500 units of enzyme per ml and digest overnight at 37°C. The DNase can then be removed by chloroform extraction.

To assay for thio-phosphate incorporation resistance to DNase digestion can be monitored using manganese containing buffer. When the phage DNA sample is run on a high resolving agarose gel the closed circular form is separated from the nicked linear form. The disappearance of the closed circular form can be monitored by incubating an aliquot with 2 units of enzyme in a ten ul reaction for 1-3 hrs. By two hrs. the closed circular form disappears in normal DNA preparations. Modified DNAs are more resistant depending on the ratio of thio-phosphate to phosphate used in the growth medium (Figure 1). The closed circular form of DNA disappears by 3 hrs. when a 50% ratio of thio-phosphate to phosphate is used compared to the persistence of the circular form when the growth medium contains 100% thio-phosphate. Other traditional methods to examine phosphorothioate nucleotides include <sup>31</sup>P NMR (Eckstein and Jovin (1983) 22:4546-4550).

## Example II

### Method of stabilizing RNA or DNA in vivo and in vitro.

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conditioned media with up to 250 mg/L of thio-phosphate added. No adverse reactions are seen in fish incubated for up to two days. Fresh media is prepared daily. Fish are sacrificed and DNA prepared from intestinal cells using DNAzol™ (Molecular Research Center, Ohio). The DNA samples are then tested for resistance to DNase digestion. Samples are incubated in a 50 ul rxn with ~ 1ug of DNA and .5 units of DNase for 0, 5, 10, 15 min. at room temp. DNA from 250 mg/L thio-phosphate media is much more resistant than wildtype DNA or DNA from 25 mg/L thio-phosphate media. Normal DNA and 25 mg/L thio-phosphate treated DNA is digested after 5 min. while 250 mg/L thio-phosphate treated DNA persists beyond 15 min. of digestion.

### Example III

#### Method of enhancing protein synthesis levels.

The stabilization of RNA by growth in thio-phosphate media can be used to enhance the protein synthesis of most proteins. The optimal level of thio-phosphate to phosphate must be determined as well absolute levels of total phosphate. Various *S. cerevisiae* strains are able to grow in minimal media containing thio-phosphate: multiploid fleischmann's yeast strain, haploid strain ATTC# 32119, and diploid strain BJ3505 (Sigma). The haploid strain *Pichia canadensis* ATTC# 14355 is less tolerant to the total amount of thio-phosphate but does grow when using yeast nitrogen based medias (YNB, BIO 101). The optimal level of total phosphate is ~1 g/L which is much less than bacteria.

Growth in all cases is enhanced by using enriched medias prepared by depleting inherent phosphate. To deplete phosphate by precipitation,  $MgSO_4$  and ammonium hydroxide are added to the media of choice. YPD ( 1% Bacto-yeast extract, 2% Bacto-peptone, 2% Dextrose) phosphate depleted media is prepared by adding 10 ml of 1 M  $MgSO_4$  and 10 ml of concentrated  $NH_4OH$  per liter and allowing the ppt. to form by leaving the solution at room temp. for 30 min. The media is then filtered and the pH readjusted to pH 6.5. Using this procedure, the detectable amount of ortho-phosphate remaining is less than ~200 mg/liter. Using phosphate levels at ~1 g/L adequate growth is observed over the full range of ratios (100% to 0%) for both *S. cerevisiae* and *Pichia canadensis*. It is best to inoculate at high densities using, for example a 1:20 dilution. Insufficient inoculum (low density) will result in poor growth.

To test for optimal protein synthesis conditions the following system can be used: a recombinant YEp plasmid containing a secreted bacterial alkaline phosphatase gene (YEpFLAG-1-BAP, Sigma; US Patents 4,703,004, 4,782,137 EP Patent 150126, and JP Patent 1983150) marked with the FLAG™ peptide DYKDDDDK. Detection of this epitope in cells is possible using immuno test strips (TagDetect kit (Stratagene). To enhance signal detection using the vector it is best to prepare spheroplasts (Yeast Cell Lysis Kit, Bio 101) before lysing cells with glass beads in phosphate buffered saline. It is possible to overwhelm the test strips with antigen and obliterate signal so various dilutions must be tested to ensure accurate results. When yeast cells (BJ3505 strain, Sigma) transformed with the expression vector are grown overnight in media containing different ratios of thio-phosphate to normal phosphate (100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 0), maximal synthesis occurs at a ~10% ratio (weight equivalent) following cessation of growth. In this regard, the optimal ratio can vary somewhat with different lots of thio-phosphate from a single manufacturer of high quality material (>90% purity) because of variable water content and phosphate contamination. While additional peaks are observed at higher ratios the growth rate is slightly reduced at these higher levels making ~10% the preferred ratio requiring less thio-phosphate. Dilution assays indicate an increase of about ten fold compared to normal media with an equivalent amount of phosphate (Figure 4).

An additional assay was developed to examine the effects of thio-phosphate media on the synthesis of secreted proteins. Yeast excrete many extracellular hydrolases and the mRNAs for these proteins are generally much more stable than other mRNAs and present at high levels. Extracellular nucleases are detected by monitoring the degradation of genomic DNA by gel electrophoresis. A two to

three fold increase of activity is observed at a 10% ratio. Ratios beyond 30% are not stimulatory and above 70% are slightly inhibitory. In contrast, lipase a much more abundant hydrolase is increased less than two fold by qualitative assays (Figure 4).

The low ratios of thio-phosphate to phosphate required for protein synthesis enhancement in yeast make it possible to simply add thio-phosphate to nutrient broths for maximum yields of protein. For any given media the level of orthophosphate should be determined (Hanna Phosphate Test Kit; Hanna Instruments). Compare with a standard phosphate solution to determine the equivalent amount of salt. Then add the appropriate amount of thio-phosphate. Decay of thio-phosphate is affected by pH (neutral to slightly basic is optimal) and temperature (4°C is optimal). For prolonged incubations (several days) it may be best to adjust the pH of the media.

Bacterial expression can be examined using the recombinant vector pFLAG-ATS-BAP (Sigma Chemical; US Patents 4,703,004, 4,782,137 EP Patent 150126, and JP Patent 1983150) marked with bacterial alkaline phosphatase. This control plasmid is expressed in the periplasmic fraction by virtue of the N-terminal OmpA signal at high levels. Enzyme activity is easily visualized using colormetric staining of whole cells. 0.5 ml of cells are pelleted and resuspended in pH 9.5 buffer (0.1 M Tris-HCL, 0.1 M NaCL, 50 mM MgCl<sub>2</sub>) along with 10 ul of NBT (nitroblue tetrazolium, 50 mg/ml) and 5 ul of BCIP (5-bromo-4-chloro-3-indolylphosphate, 50 mg/ml). After sufficient time (there may be a slight delay in the activity of induced cells) the samples are compared and then centrifuged to compare pellets. Various ratios of modified phosphate to phosphate are examined: 0%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60, 70%, 80%, 90% and 100%. Because the protein is already present at high levels further induction results in the formation of inactive inclusion bodies. To determine the optimal ratio a lower level of lactose is used in the media to prevent the formation of inclusion bodies during the analysis (M9 minimal media made with .1% rather than .5% lactose and no glucose, total phosphate levels of 10 g/L). The host strain employed is JM109 and no activity is observed in bacterial cells without the pFLAG-ATS-BAP expression vector several hours (4-7) after induction in lactose containing media. At low lactose levels the optimal ratio is identified at a 50% ratio of modified phosphate to phosphate and is elevated over controls several fold (Figure 4). At higher levels of lactose a reduction in the optimal ratio of thio-phosphate to phosphate delays the onset of inclusion bodies and enhanced enzyme activity is apparent at lower ratios (10-20%).

A recombinant vector containing the beta-galactosidase gene (Promega's TA vector) can also be examined. Enzyme activity is detected via a colormetric assay. Cells are grown in minimal media supplemented with 2% lactose for optimal induction of beta-galactosidase. After several (~4-5) hrs bacterial cells are removed to assay for enzyme activity. To .5 ml of cells add .5 ml of 200 mM Tris-HCL, pH 7.5 and 5 ul of 1% SDS (sodium dodecyl sulfate). Then add 10 ul of IPTG (100 mM) and 20 ul of X-gal (50 mg/ml), mix and incubate at 37°C. The enzyme is inhibited at low ratios of thio-phosphate to phosphate (5-30%) and recovers from inhibition at higher ratios (50-70%). In the presence of glucose which represses the enzyme, the activity is much lower but can be enhanced 10 fold by using a 50% ratio of thio-phosphate to phosphate.

In bacteria as in yeast more stable mRNAs are diluted upon stabilization of less stable mRNAs. Total protein synthesis in bacteria and yeast indicates that soluble protein synthesis levels are not enhanced at low ratios of thio-phosphate to phosphate (10-30%). At higher ratios a slight decline in growth appears to account for the reduction in total protein synthesis detected. To measure protein synthesis 4 ml of cells are pelleted washed in PBS and resuspended in lysozyme solution for bacteria. Cells are then pelleted again and washed in PBS to remove lysozyme. Cells are then lysed in 400 ul of 1% SDS and .2 N NaOH. The extract is then diluted in water to 2 ml and assayed using the biuret reagent (Gornall et al (1949) J. Biol. Chem. 177:751). Yeast are assayed without lysozyme.

#### Example IV

##### Method of enhancing the natural rate of mutagenesis.

Recombinant M13 phage DNA can be mutated at a high rate by amplification in thio-phosphate containing media. Mutation rates are assayed easily by scoring plaques for beta-galactosidase activity using a standard blue/white colormetric assay involving IPTG (isopropyl-beta-D-thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside). To assay for activity, an aliquot of phage is mixed with .2 ml of JM109 cells, 10 ul IPTG (100mM), and 50 ul Xgal (2%). Three mls of top agar are added and the entire mixture with phage is plated on 1X YT (bacto tryptone, 8 g/L; bacto yeast extract, 10 g/L; NaCl, 10 g/L. Adjust pH to 7.5) plates and incubated overnight. Wildtype plaques are blue and those with mutations are colorless. After one round of phage amplification in thio-phosphate media, the bacterial cells are spun out and the supernatant assayed for colorless plaques. The phage containing supernatant is plated such that ~1000 plaques are formed per plate; the titer is similar for wildtype and phosphorothioate phage after one round of amplification. For phosphorothioate DNA phage approximately two mutant plaques are observed per plate or 1/400. Wildtype phage grown for the same period do not generate mutants (less than 1/4000). The percentage of mutants observed can be increased by another round of amplification in thio-phosphate media. The phosphorothioate DNA phage from the first amplification are used to inoculate the second round of amplification. The phage supernatant from the second round of amplification have a lower titre (~five fold) than that of similarly prepared wildtype phage. Nevertheless, the mutation rate is quite high, on the order of 1/6 compared with wildtype phage grown under similar conditions (<1/1000). The fold enhancement (~>200) of the mutation rate is much greater than expected from in vitro studies (20 fold) indicating that multiple repair mechanisms are inhibited (Kunkel et al (1981) PNAS 78:6734 -6738).

*S. cerevisiae* grown in thio-phosphate media (phosphate free minimal media, EMM supplemented with SP, see above and 1-10 g/L thio-phosphate) also exhibit an enhanced mutation rate though not as great as the fold enhancement observed in bacteria. To test the mutation rate yeast cells were selected for canavanine resistance (60 ug /ml) in minimal media minus arginine (Hoffman (1985) J. Biol. Chem. 260:11831-11836) and 100% thio-phosphate. An overnight culture of the haploid yeast strain ATTC 32119 was used to inoculate thio-phosphate media at a one to twenty volume ratio. The number of viable yeast cells after one to two days of growth at 30°C is reduced compared to wildtype when tested on normal phosphate containing plates. The yeast are pelleted by centrifugation and washed with water and resuspended in water at the original volume. The resuspended cells are then spread (~50 ul 100 mm plate) on canavanine containing plates and Can<sup>R</sup> colonies observed after two days as distinct colonies. The average increase in mutation rate for thio-phosphate media compared to normal media is approximately 10 fold (range observed 3.9-18.5).